Ammonia and Carbon Dioxide: Quantitation and Electroantennogram Responses of Caribbean Fruit Fly, *Anastrepha suspensa* (Diptera: Tephritidae)

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ABSTRACT A Fourier transform infrared (FTIR) spectroscopic method was developed to quantify ammonia (NH_3) and carbon dioxide (CO_2) to facilitate accurate determination of antennal sensitivity of adult Caribbean fruit flies, *Anastrepha suspensa* (Loew), to these compounds. Electroantennogram (EAG) recordings were made from antennae of flies that were exposed to known quantities of each of the pure gases and to mixtures of these gases. EAG response to volumetric dilutions of saturated vapor from a commercially available ammonium bicarbonate lure was determined, and FTIR analysis was used to measure the amount of NH_3 and CO_2 in the samples. Maximal EAG responses were elicited with doses $\geq$24 μg NH_3 and $\geq$57 μg CO_2. Mean female response was greater than male response to substrates that included CO_2. For both sexes, EAG response to NH_3 was greater than response to CO_2. When NH_3 and CO_2 were combined either as a mixture of pure gases or as vapor emitted from the lure, the EAG response was approximately equal to the sum of the individual responses to the two compounds. A polynomial regression was the best fit for describing antennal response to ammonia, carbon dioxide, and mixtures of these chemicals whether obtained from pure gas sources or from commercial lures.

KEY WORDS *Anastrepha suspensa*, Tephritidae, electroantennogram, ammonia, carbon dioxide

TEPHRITID FRUIT FLIES INCLUDE a number of economically important agricultural pests worldwide, and much attention has been directed toward the development of effective lures for detecting and monitoring their populations. The Caribbean fruit fly, *Anastrepha suspensa* (Loew), occurs in Florida, is a quarantine pest of citrus, and poses an additional threat to several other tropical and subtropical fruits (Greany and Riherd 1993). In research comparing sugar-based fruit fly attractants, it was determined that protein impurities were responsible for fruit fly attraction when the odor of ammonia was noted from a number of test preparations (McPhail 1939). Like other tephritids, *A. suspensa* adults require protein meals for the completion of reproductive maturation (Bateman 1972, Landolt and Davis-Hernandez 1993), and traps baited with liquid protein solutions or synthetic ammonia have been used for *Anastrepha* spp. as well as other tephritid fruit flies. Various formulations of synthetic ammonia have been used as baits for fruit flies, including ammonium acetate (Prokopy 1968, Moore 1969), ammonium carbonate (Liquido et al. 1993), ammonium bicarbonate (Robacker and Warfield 1993), and ammonium hydroxide (Stills 1964, Boucher et al. 2001). Research on the Mexican fruit fly, *Anastrepha ludens* (Loew), and the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), found that additional components such as putrescine, methylamine, and trimethylamine enhance the efficacy of traps baited with synthetic ammonia (Robacker and Warfield 1993, Heath et al. 1995, Heath et al. 1997). Commercial formulations of ammonium acetate (Suterra, Bend, OR) and ammonium bicarbonate (Agrisense-BCS, Mid Glamorgan, UK) are available for use in fruit fly traps.

Documentation of the role of ammonia was provided by Mazor et al. (1987), who used dilutions of a pure ammonia solution to obtain a direct correlation between capture of female *C. capitata* and ammonia concentration. This has also been observed in field tests, with captures of *C. capitata* increasing as ammonia release rate was increased by varying amounts of borax (sodium tetraborate decahydrate) added to the aqueous protein bait Nulure (Heath et al. 1994). A direct relationship between ammonia release rate and *A. suspensa* capture was observed in similar laboratory choice tests, but when tested in the field, the relationship was more variable (Epsky et al. 1993). In subsequent tests with other *Anastrepha* spp., results of field comparisons of traps baited with synthetic ammonia-based lures versus liquid protein baits have

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been highly variable and again capture was not correlated with relative ammonia release rates (Thomas et al. 2001, Epsky et al. 2005). Because of the need for improved lures for the Caribbean fruit fly and for other pest *Anastrepha*, research was initiated to determine the role of ammonia release rate for use in traps for these fruit flies.

Keiser et al. (1976) found that acetic acid, which is released from ammonium acetate along with ammonia, is attractive to *C. capitata.* Similarly, carbon dioxide is released from ammonium bicarbonate lures along with ammonia. Electroantennogram (EAG) analysis, used to investigate olfaction of the Queensland fruit fly, *Bactrocera tryoni* (Froggatt), found antennal response to both ammonia and carbon dioxide (Hull and Cribb 2001a). Thus, in addition to differences in dosage, source of ammonia used in a synthetic lure may provide additional chemicals that may be either attractive or repellent to a target fruit fly, either of which may affect lure efficacy. Antennal responses are a prerequisite for behavioral responses, making EAG a useful tool for screening potential attractants. Therefore, the first step of this research was to assess antennal response of adult *A. suspensa* to ammonia and carbon dioxide and to quantify antennal sensitivity to these chemicals alone and in combination.

Accurate quantification of ammonia is difficult because of its corrosive nature. Previously reported techniques (Heath et al. 1995, Robacker and Bartelt 1996) result in considerable variation, which compromises quantification. We investigated the use of Fourier transform infrared (FTIR) spectroscopy as a method for quantification of ammonia and carbon dioxide. This enabled us to deliver quantified amounts of ammonia and carbon dioxide as well as mixtures of the two gases to measure the dose-dependent EAG response of *A. suspensa.* The amounts of ammonia and carbon dioxide released from a commercial ammonium bicarbonate lure were then determined using FTIR, and EAG response of flies to the ammonium bicarbonate lure was evaluated.

Materials and Methods

Infrared Sampling and Analysis. FTIR spectroscopic methods were used to determine the quantities of ammonia and carbon dioxide. The system consisted of a Thermo Nicolet Magna 550II FTIR spectrometer (Madison, WI) equipped with a mercury cadmium telluride (MCT-A) detector, a KBr beamsplitter, and a 2-m (200 ml) gas cell with a thermal jacket (Thermo Electron Corp., Waltham, MA) and ZnSe windows. The detector was cooled with liquid N_2, and the gas cell was heated to 100°C, with a vacuum of −26.5 Hg. Spectra were obtained by subtracting background from 128 scans of the sample in the range between 4,000 and 700 cm^{-1}. The method consisted of quantitation of one band for ammonia analysis (997–987 cm^{-1}) and one band for carbon dioxide analysis (725–715 cm^{-1}). FTIR calibration curves were generated using six known quantities of anhydrous ammonia gas (99.99% pure, 1.5–48.2 μg) and five known quantities of carbon dioxide (99.8% pure, 0.9–4.6 μg; Aldrich, Milwaukee, WI).

For chemical analysis, a commercial ammonium bicarbonate lure (AgriSense-BCS) was placed in a gas-tight jar with a septum port in the lid. A 24-h equilibration time was allowed for the headspace to saturate with ammonium bicarbonate vapor at room temperature (24°C). Volumetric two-fold dilutions of saturated vapor were prepared that corresponded to the series of samples used in EAG studies (see below). Sample dilutions were prepared at room temperature (24°C) and ambient pressure in 1-liter gas-tight stainless steel cells containing a vent valve on one end and a septum port on the opposite end. Cells were filled with air purified with a Whatman 75–52 FTIR purge gas generator ($CO_2 < 1$ ppm [Whatman, Tewksbury, MA]), and a Hankison HIT-20 air dryer (Hankison, Canonsburg, PA) was used for water removal. Dilutions were prepared by first extracting the desired volume of air from the cell and then substituting it with the corresponding volume of ammonium bicarbonate saturated vapor. Using a gas-tight syringe (VICI Precision Sampling, Baton Rouge, LA), an aliquot of 50 ml was drawn from the corresponding metal cell and injected into the evacuated FTIR cell. FTIR analysis was performed as described above, and results were processed using TQ Analyst, version 6 (Thermo Electron Corp.). From the FTIR calibration curves, quantities (in micrograms) of ammonia and carbon dioxide were calculated for each dilution. Final quantitative determinations were mean values based on three replicates.

2-Butanone (Aldrich) was used as the reference standard in all tests. This compound was chosen because it had been reported to elicit a strong EAG response in *B. tryoni* (Hull and Cribb 2001a), and our initial tests showed a strong response in *A. suspensa* as well (PK, unpublished data). 2-Butanone was analyzed using a Trace gas chromatograph (Thermo Electron Corp.), equipped with a large volume on-column injector (LVOC septum injector) and flame ionization detector (FID). An MXT-WAX column, 30 m by 0.53 mm by 0.5 μm (Restek Corp., Bellefonte, PA) was used for analysis with helium as the carrier gas at a linear flow velocity of 18 cm/s. An initial oven temperature of 60°C was held for 3 min, followed by temperature programming of 1°C/min to 70°C, and then increased at 20°C/min to 180°C. The final temperature was held for 1.5 min. Detection of the 2-butanone was done with a flame ionization detector. Detector temperature was 210°C. The chromatographic data were collected and processed using ChromQuest software, version 2.53 (Thermo Electron Corp., Waltham, MA). 2-Butanone was determined to be >99% pure, and further confirmation of the identity of 2-butanone was obtained by matching the IR spectra with published spectra.

Insects. Caribbean fruit flies were obtained from a laboratory colony maintained at the USDA-ARS, Subtropical Horticulture Research Station, Miami, FL. Adult flies were housed in screen cages (30 by 30 by 30 cm) and given unlimited access to water (from agar

blocks) and adult food (4:1 mixture of refined cane sugar: protein hydrolysate). Rearing conditions consisted of a 12:12 h (L:D) photoperiod, 70% RH, and ambient room temperature. All flies for EAG studies were collected from mixed-sex cages and were sexually mature, ranging in age from 8 to 14 d posteclosion.

EAG Analysis. EAG signals were recorded with a Syntech EAG system (Hilversum, Netherlands), which included a probe/micromanipulator (MP-15), a data acquisition interface box (serial IDAC-232), and a stimulus air controller (CS-05). Fresh antennal preparations were mounted between pipette electrodes using a small amount of salt-free electrode gel (Spectra 360; Parker Laboratories, Fairfield, NJ) applied to the tip of each pipette. The electrodes were drawn from thin-walled glass capillaries (1.5 mm o.d., World Precision Instruments, Sarasota, FL), filled with 0.1 N KCl, and positioned over silver wires (0.5 mm, World Precision Instruments) on the Syntech probe. Electroantennal signals were collected and analyzed with the Syntech EAG 2000 program on a personal computer.

A stream of humidified air, purified with activated charcoal, was passed continuously over the antennal preparations at a flow rate of 400 ml/min, with the tip of the delivery tube placed 1 mm from the antenna. Using gas-tight syringes (VICI Precision Sampling), EAG samples were manually injected (0.5 s) into the airstream through a port in the delivery tube 13 cm from the antennal preparation. In each experiment, the antenna was first presented with a negative control, consisting of an injection of clean air equal in volume to the sample injections. This was followed by injection of the standard reference compound, 20 μl of saturated headspace from the container of 2-butanone, and then with injections of test samples. For the pure gases, three sets of samples were tested separately: ammonia, carbon dioxide, and a 1:1 molar mixture of the two gases. Each set consisted of a series of six two-fold dilutions (3,906.25–125,000 ppm [vol:vol] prepared in 250-ml gas-tight glass cells; Analytical Research Systems, Gainesville, FL), using the same method used to prepare FTIR vapor samples in stainless steel cells. Injection volume was held constant at 500 μl for all samples of pure gases. For the ammonium bicarbonate–saturated vapor, a range of doses was obtained with a series of six two-fold increases in headspace sample volume (0.125–4 ml). Test chemicals were presented in ascending order from the lowest to the highest dose. There was a 2-min interval between injections to prevent adaptation of the antennae. Stimulation with the 2-butanone standard was repeated at regular intervals throughout the experiment to assess the decline in antennal response over time. EAG responses were initially measured in millivolts (peak height of depolarization) and converted to normalized responses using the Syntech EAG 2000 software. Normalization corrected for time-dependent variability in antennal performance, and test responses were expressed as a percentage of the standard response. Any response measured with the negative control was subtracted from the normalized response. The final corrected, normalized values were used for analyses.

Statistical Analysis of EAG Results. EAG response per substrate concentration was determined from three subsamples per individual at each dose, and the mean responses from five individuals were used for analysis for each substrate. Regression analysis was used to describe the relationship between antennal response and substrate concentration (SAS Institute 1985) for each test substrate and each sex. Several regression models were tested including linear, logarithmic, polynomial, and hyperbolic models. SigmaPlot 8.0 (SPSS, Chicago, IL) was used to graph EAG response curves based on the optimal model obtained from regression analysis. Significant differences in slopes and intercepts were tested using analysis of covariance (ANCOVA), and differences in intercept were identified using Fisher protected least significant difference test ($P = 0.05$). Separate comparisons were made between the two sexes for each substrate and for ammonia and carbon dioxide alone versus the mixture within each sex.

Results

FTIR analysis resulted in linear regressions (r^2 = 0.925, y = −0.0523 + 0.0042x and r^2 = 0.982, y = −0.5610 + 0.00204x) for ammonia and carbon dioxide, respectively (where x is substrate quantity expressed in micrograms and y is peak area). Antennal response was obtained with either ammonia or carbon dioxide; however, the higher doses of carbon dioxide were too large to be quantified directly by FTIR. Therefore, quantities of carbon dioxide delivered to the antennae in EAG were determined by extrapolation from standard curves. Amounts of ammonia delivered were 0, 1.5, 3, 6, 12, 24, and 48 μg; amounts of carbon dioxide delivered were 0, 3.6, 7.2, 14.3, 28.7, 57.4, and 114.7 μg for tests of single chemicals and equimolar mixture.

Polynomial regression models were the best fit of the EAG responses of both females (R^2 = 0.740; y = 0.71 + 3.52x − 0.04x^2; error mean square (EMS) = 179.37; x is test substrate quantity in micrograms, y is the average normalized percent EAG response compared with the standard 2-butanone) and males (R^2 = 0.938; y = 2.36 + 3.11x − 0.04x^2; EMS = 23.7) to ammonia, and there were no differences in either intercept or slopes between the sexes (Fig. 1). Polynomial regression models were also the best fit of the EAG responses of both females (R^2 = 0.614; y = 4.46 + 0.85x − 0.005x^2; EMS = 87.36) and males (R^2 = 0.696; y = 2.11 + 0.56x − 0.003x^2; EMS = 27.81) to carbon dioxide (Fig. 2). There were no differences in slopes, but there was a higher intercept in the EAG response of females ($F = 14.67$; df = 1,64; $P = 0.0003$). When the stimulus was presented as an equimolar mixture of ammonia and carbon dioxide, results again fit the polynomial model for both females (R^2 = 0.924; y = 1.79 + 5.75x − 0.08x^2; EMS = 111.98) and males (R^2 = 0.910; y = 4.18 + 3.88x − 0.05x^2; EMS = 60.53). In tests with the mixtures, however, there were both differences in slopes ($F = 21.79$; df = 1,64; $P < 0.0001$

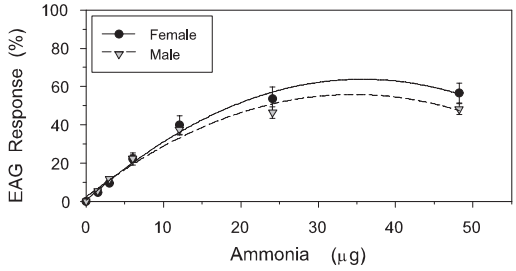

Fig. 1. Mean EAG response and polynomial regression of male and female Caribbean fruit flies to pure ammonia gas. EAG response depicted as percentage of the standard response (2-butanone reference). Bars indicate SE; $n = 5$ per sex.

for β_1 and $F = 5.84$; df = 1,64; $P = 0.0185$ for β_2) and intercept ($F = 27.06$; df = 1,64; $P < 0.0001$) between the two sexes, with a higher EAG response in females (Fig. 3).

Separate ANCOVA compared antennal response to ammonia and carbon dioxide alone versus a mixture of ammonia plus carbon dioxide for females and for males. There were no differences between response (as indicated by intercept, mean ± SD) of males to ammonia alone versus the mixture (24.5 ± 19.00 versus 32.6 ± 25.12%; $F = 0.47$; df = 1,64; $P = 0.4934$). However, there were higher responses by females to the mixture (44.1 ± 37.25%) than to ammonia alone (26.6 ± 25.48%; $F = 35.83$; df = 1,64; $P < 0.0001$) or to carbon dioxide alone (18.5 ± 14.60%; $F = 115.67$; df = 1,64; $P < 0.0001$), and there was a higher response by males to the mixture (32.6 ± 25.12%) than to carbon dioxide alone (11.5 ± 9.28%; $F = 175.87$; df = 1,64; $P < 0.0001$). There was no difference in slope of response of males to ammonia alone versus the equimolar mixture, but there were differences among all other combinations.

FTIR analysis of the commercial ammonium bicarbonate lure determined that saturated headspace contained an average of 12.0 ± 0.83 (SD) μg /ml of ammonia and 15.2 ± 0.64) μg/ml of carbon dioxide ($n = 3$). For the saturated vapor from the ammonium bicarbonate lure (Fig. 4), polynomial regression models were again the best fit to the EAG response, where x is test substrate amount in microliters and y is the average normalized percent EAG response compared with the standard 2-butanone for females ($R^2 = 0.800$; $y = 12.62 + 0.05x - 0.001x^2$; EMS = 174.47) and for males ($R^2 = 0.871$; $y = 10.55 + 0.04x - 0.001x^2$; EMS = 81.08). As was observed in response to carbon dioxide, there were no differences between slopes, but the intercept for the females was higher than for males ($F = 4.47$; df = 1,64; $P = 0.0384$).

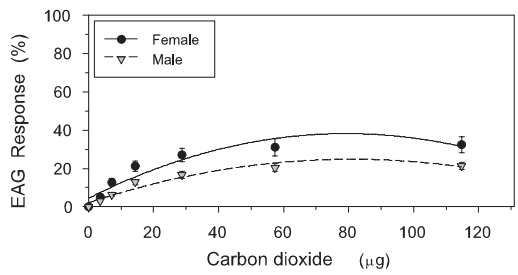

Fig. 2. Mean EAG response and polynomial regression of male and female Caribbean fruit flies to pure carbon dioxide gas. EAG response depicted as percentage of the standard response (2-butanone reference). Bars indicate SE; $n = 5$ per sex.

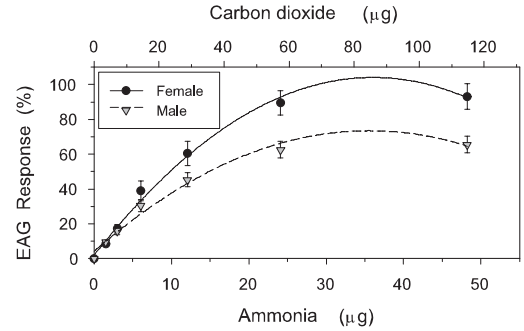

Fig. 3. Mean EAG response and polynomial regression of male and female Caribbean fruit flies to an equimolar mixture of pure gases of ammonia and carbon dioxide. EAG response depicted as percentage of the standard response (2-butanone reference). Bars indicate SE; $n = 5$ per sex.

Discussion

FTIR provided an accurate and rapid method for quantification of ammonia compared with previous results using an ammonia-specific ion-selective electrochemical probe (Heath et al. 1995 and references therein). For ammonia determinations with an ion-selective probe, the test substrate is placed in an Erlenmeyer flask and the flask is purged for 1 h with an air flow of 1 liter/min. Volatiles are directed to a sparge system that consists of a gas dispersion tube placed in a graduated cylinder containing an HCl solution (0.05 N). After a collection, the ionic strength of the sample solution is adjusted to pH of 9.0 using

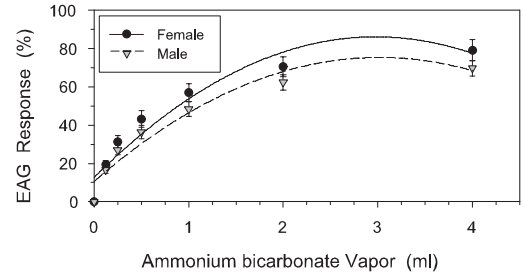

Fig. 4. Mean EAG response and polynomial regression of male and female Caribbean fruit flies to saturated vapor released from a commercial ammonium bicarbonate lure. EAG response depicted as percentage of the standard response (2-butanone reference). Bars indicate SE; $n = 5$ per sex.

NaOH/0.05 M disodium EDTA/10% methanol containing a color indicator for pH. A standard ammonium calibration curve has to be prepared each day an analysis is done. Using FTIR, no sample preparation is needed other than dilution. Interestingly, with the FTIR method, higher accuracy was obtained for carbon dioxide rather than ammonia. Overall, the FTIR method allows for rapid analysis of pure ammonia and carbon dioxide, and methods used for FTIR determination can also be used to prepare quantitative mixtures of these gases in a facile manner.

We hypothesized that antennal response to ammonia would be higher for females than for males because of the greater need for protein by females for egg development (Landolt and Davis-Hernandez 1993). Presumably, the increased need for protein would be reflected in increased numbers of antennal receptor neurons sensitive to volatile by-products of protein degradation, and consequently, in an increased physiological response (e.g., Arn et al. 1975, Mayer et al. 1987). In field tests, captures of *A. suspensa* in traps containing liquid protein baits typically have a strong female bias, with female:male ratios of 2:1 (Calkins et al. 1984). Sex ratio in capture of *A. ludens* and *C. capitata* have been found to vary from male-biased to female-biased depending on host and season of the field trials (Thomas et al. 2001, Epsky et al. 1999). Factors such as reproductive status, time period of food deprivation, and difference between wild and laboratory-reared flies can also affect response and sex ratio of captured flies (Heath et al. 1995, Robacker 1999). All the flies used in our studies were fully fed, sexually mature, laboratory-reared flies that were obtained from mixed sex cages and were probably mated. Additional studies are needed to fully assess the influence of these factors on antennal response for both male and female flies.

Among all the substrates tested in this study, only substrates that included carbon dioxide resulted in a higher antennal response by females. Stange (1999) proposed that carbon dioxide is a short-range oviposition attractant for *B. tryoni,* that small lesions in the skin of host fruits are sources of carbon dioxide release, and that this emission attracts gravid females to suitable oviposition sites. The higher response of female *A. suspensa* is apparent at low doses, suggesting that females may be able to detect small fluctuations in carbon dioxide concentrations, such as those occurring in close vicinity of their host plants (Stange 1997). Working with the same species, Hull and Cribb (2001a) used EAG to investigate the number of receptor neuron types involved in olfaction of ammonia and carbon dioxide, among other volatile compounds. They concluded that *Bactrocera* antennae have at least three distinct receptor types for detection of the seven chemicals examined and that ammonia and carbon dioxide were detected by separate receptors. The authors also did a more thorough investigation of the carbon dioxide receptor using single-cell electrophysiological recordings (Hull and Cribb 2001b). They estimated that only ≈10% of the total olfactory sensilla function in carbon dioxide reception (≈270 CO_2 sensilla per 2,500 total sensilla per female antenna). Their studies were conducted with female flies only. Although male *A. suspensa* antennae responded less to carbon dioxide than females, an antennal response was obtained when exposed to carbon dioxide. Additional studies are needed to see if antennal response by either females or males is indicative of increased attraction to carbon dioxide in a lure such as ammonium bicarbonate. Field trials of a number of *Anastrepha* species have found that, generally, traps with ammonium acetate plus putrescine outcapture traps baited with ammonium bicarbonate plus putrescine (Epsky et al. 2005), although higher capture of *A. ludens* has been obtained with ammonium bicarbonate-baited traps (Robacker 1999). Carbon dioxide is well known as a host-seeking cue for hematophagous flies, such as mosquitoes (Bowden 1991) and tsetse flies (Willemse and Takken 1994), but little is known about its role in tephritid host orientation.

Differences were also observed between EAG responses to the ammonia:carbon dioxide combination presented as a quantified mixture versus as vapor from a commercial ammonium bicarbonate lure. The primary difference between the two formulations was in the ratio of the components. We expected the ammonium bicarbonate lure to release ammonia and carbon dioxide in equal molar amounts and prepared our mixture with the appropriate ratio, ≈30% ammonia and ≈70% carbon dioxide. However, quantitation of saturated vapor from the commercial lure revealed ≈44% ammonia and ≈56% carbon dioxide. With both formulations, there was a higher antennal response in females compared with males; but with the equimolar mixture, there was also a difference in slopes of the response curves, with a greater slope for female response. It is not known if the difference observed with the two formulations is caused by the ratio differences or by the presence of unknown impurities from the ammonium bicarbonate lure. However, given samples of the two formulations containing equal amounts of ammonia, the equimolar mixture would contain more carbon dioxide than the lure, and it is the carbon dioxide component that elicits significantly different EAG responses between male and female flies. This suggests that the ratio differences would contribute, at least in part, to the differences in slopes observed between male and female response curves. Thus, in addition to dose, the ratio of components may be critical in developing effective food-based lures.

The long-term goal of this research is to develop improved synthetic lures for capturing economically important fruit flies, especially pest *Anastrepha.* This objective may be achieved through an approach that examines the physiological basis for attraction to food-based lures by comparing EAG response between and among putative attractants within and among tephritid species. EAG technology has been used to detect chemoreceptive response to plant volatiles (from leaves and fruits) in *C. capitata* (Light et al. 1988) and the Oriental fruit fly, *Dacus dorsalis* Hendel (Light and Jang 1987) and to other behavior-

ally active chemicals, including ammonia, in *B. tryoni* (Hull and Cribb 2001a). EAG analysis examines the physiological mechanisms involved with the initial reception and transduction of olfactory signals. There is not necessarily a direct correlation between level of EAG response and behavior; for example, high doses of olfactory stimulants may result in a loss of attraction or possibly a switch to repellency. EAG studies must be complemented with behavioral bioassays and field tests to fully understand the relationship between antennal response and subsequent insect capture. Efficacy of food-based synthetic attractants for pest fruit flies may be enhanced by optimizing the release rates and ratios of the individual components of the lure. A better understanding of the relationship between EAG response and attractiveness of synthetic lures may facilitate the development of improved attractants for the Caribbean fruit fly and other pest tephritids.

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